PATENT SPECIFICATION (11) 1 557 722

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(21) Application No. 31855/76 (22) Filed 30 Jul. 1976

(31) Convention Application No. 603190 (32) Filed 8 Aug. 1975 in

(33) United States of America (US)

(44) Complete Specification Published 12 Dec. 1979

(51) INT. CL.[2] G02B 21/34 //
G01N 33/16

(52) Index at Acceptance
G2J 8G
G1B BR

(72) Inventor: JOHN AUTEN GEATING

(54) IMPROVEMENTS IN SLIDES

(71) We, GENERAL ELECTRIC COMPANY, a corporation organized and existing under the laws of the State of New York, United States of America, of 1 River Road, Schenectady 12305, State of New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

This invention pertains to the making of stained preparations of biological specimens, particularly of blood.

It has been known in the past, see for example U.S. Patent 3,389,052, of Ehrenreich et al., that protection of cytological specimens can be obtained by arranging the specimens in the form of smears upon a slide for shipment to a pathological laboratory, and where a protective coating is removed prior to or as part of a conventional staining procedure.

For such an approach, an aerosol spray is used to apply polyethylene glycol as a coating. This coating would be removed in the usual staining process. Also known in the prior art is the use of other coatings which can be specifically removed prior to staining of the specimen.

Also known is the use of a cover slip, precoated with an adhesive, which when applied to a microscope slide wet with a tissue clearing fluid such as xylene or toluene, becomes sufficiently adhesive or tacky to be bonded firmly to the slide by being pressed down upon it. It is incidental that the specimen upon the slide has usually been stained; such a teaching is purely one of mechanical fixing of the cover slip. See for example U.S. Patent 3,498,860 - Pickett.

Yet another previously known technique is the coating of a microscope slide with a transparent metallic fluoride to produce adhesion of a histological specimen such as a frozen or paraffin-cast section. Such technique can use an evaporated layer of magnesium fluoride a fraction of a wavelength of visible light thick. The virtue of such a technique is that it appears unaffected by any ordinary tissue processing, and is itself not a culture medium. No relation to staining is even suggested. See in this regard U.S. Patent 3,770,477 - Weichselbaum.

Still another approach used in the past is the production of easily measured small quantities of dry reagents by saturating absorbent inert carriers of fibrous material in sheet form with a solution of known concentration of the reagent which can be uniformly distributed throughout the carrier and permitted to dry. This permits the carrier to be cut into portions which are any fraction of the total carrier and thus provide a definite portion of the reagent. For example, 10 milligrams in slution distributed over 100 square centimeters can provide 100 micrograms in one square centimeter of carrier. Such a small but precisely determined quantity may be recovered in solution by immersing the carrier portion in a solvent. The idea of carrying dry reagents in a carrier strip is extended to a method of staining dried blood smears. Whatman #1 filter paper (Whatman is a Trade Name) can be saturated with a methanol solution of eosin Y, methylene blue, Azure A, and methylene violet, and dried. For use, the dried stain-carrying paper is immersed in a methyl alcohol solution of a catalyst such as propylene glycol or chlorophyl. It is then placed over the dried blood smear, left on it from one to three seconds, and then removed. The stained surface (ordinarily a microscope slide) is then rinsed in a buffer solution, and dried. The staining operation itself is stated to be rapid--of the order of 20 to 90 seconds. Other bacteriological type stains may be applied similarly. In any case, the paper strip is used simply as a carrier of

stains which must be brought into solution by wetting the strip before use; and the strip is removed from the preparation after is has served its purpose of bringing the stain solution to the specimen. Relative to the above, see U.S. Patent 3,678,151 - Horonick et al.

Also treated in the prior art is the problem of preventing samples such as those of diarrhetic stools, which are coated with polyvinyl alcohol fixative or similar material such as ethyl cellulose, from being loosened from the slide to which they are bonded by the methyl alcohol in which they are repeatedly immersed in the staining process. This has been accomplished by immersing the slide, prior to the staining process, in water, saline solution, glycerol, diethylene glycol, or saline solution. Alternatiely, slides may be coated with neoprene; or they may be roughened prior to use. However, this technique is directed to the use of organic plastic encapsulation as a carrier of stain or a protector of stained samples; and is concerned with protecting an encapsulating mount from attach by the stain solution, subsequently applied, see U.S. Patent 3,737,335 - Feinberg.

Also known in the prior art is the preparation of a prestained slide carrying a dried mixture of methylene blue NN and cresylviolet acetate stains. When a drop of blood is placed on the slide and spread with a cover slip, it soon becomes differntially stained in a manner ordinarily requiring two separate preparations, See U.S. Patent 3,796,594.

Finally, it is also known in the prior art to use prestained slides as described above to detect malarial parasites in the blood, see U.S. Patent 3,834,874.

The present invention provides a method of staining and sealing a biological sample air-dried and affixed to a microscope slide which comprises the steps of:

a) providing a viscous fluid formulation of a transparent resin selcted from the group consisting of : methyl cellulose; polyvinyl pyrrolidone and methyl cellulose; ethyl cellulose; carboxymethyl cellulose; hydroxyethyl cellulose; hydroxypropyl cellulose; polyoxyethylene resins: and a biological stain in a solvent mixture comprising water;

b) applying a quantity of the viscous fluid formulation to an area of the affixed biological sample smaller than the area of a cover slip;

c) applying the cover slip to the applied quantity of viscous fluid formulation on the slide;

d) leaving the cover slip undisturbed upon the said fluid formulation n the slide to permit the surface tension of the fluid to draw the cover slip against the sample and the slide, whereby the fluid formulation is spread over the portions of the sample and the slide which are covered by the cover slip, and the stain dissolved in the fluid formulation is brought into contact with the sample and stains it.

The present invention also provides a viscous fluid formulation for the simultaneous staining and sealing to a coverslip of a biological sample affixed to a microscope slide comprising:

a) a transparent resin selected from the group consisting of methyl cellulose; polyvinyl pyrrlidone and methyl cellulose; ethyl cellulose; carboxymethyl cellulose; hydroxyethyl cellulose; hydroxyproply cellulose; polyoxyethylene resins

b) a biological stain

c) a solvent comprising water.

Upon a biological sample, e.g. blood as an air-dried smear affixed to a standard glass microscope slide, prepared in the conventional fashion of the prior art, there is deposited from a flexible bottle equipped with a bozzle ("squeeze bottle") or similar dispenser, a strip or bead of a viscous liquid formulation containing a transparent resin such as methyl cellulose, in which the appropriate stain is dissolved. A clean glass cover slip is placed over the slide in contact with the viscous liquid bead, and the surface tension of the liquid draws the cover slip down against the slide and its smear, spreading the liquid over the slide in the process, and bringing the solubilized stain into contact with the sample. No further action is required of the operator. The sample rapidly becomes stained. The volatile solvents in the viscous liquid evaporate, leaving the resin solid as a transparent encapsulating matrix which holds the cover slip rigidly upon the slide, and seals the sample from the ambient atmosphere, preversing it.

This procedure is economical of the operator's time, particularly where, as in a pathological laboratory, large numbers of sample are to be stained with the same stain.

The present invention will be further described by way of Example only, with reference to the accompanying drawings in which:-

Figure 1 represents a microscope slide with a dried sample upon it and a length of viscous formulation upon it.

Figure 2 represents the microscope slide of Figure 1 with a cover slip in proper position to be placed upon it.

Figure 3 represents the microscope slide and cover slip of Figure 2, after the cover slip has been placed in contact with the viscous formulation and has been drawn down upon the slide and the dried sample.

Example 1. A 5 percent nonaqueous solution of methyl cellulose is prepared by adding to 100 milliliters of N, N,-dimethyl formamide, with stirring, 5 grams of hyd-

roxypropyl methyl cellulose powder, nominal viscosity 15 centipoises. Dow Chemical Company, Midland, Michigan, 48640, is a supplier of this material under its designation A-15. To 1.0 milliliter of the solution so prepared, there is added 0.5 milliliters of a solution of 1.0 gram of Wright Stain Powder in 20 milliliters of distilled water. Wright Stain Powder is a commercially available product, furnished commission certified, which is a convenient form of the solids which, in solution, constitute Wright's stain. There is then added to the methyl cellulose Wright's stain mixture 0.5 milliliters of reagent grade glycerine.

Methyl cellulose buffered stock solution is required for the next four examples. Its preparation is therefore described separately:

To 82.35 milliliters of 0.2 molal disodium orthophosphate (Na_2HPO_4) is added 17.65 milliliters of 0.177 molal citric acid ($C_6H_8O_7$) (this combination is known in the art as McIlvaine's standard buffer); the mixture is then heated to 90 to 95 degrees C. (In the proportions given, this buffer produces approximate neutrality, and references herein to McIlvaine's standard buffer will mean a solution of substantially these proportions.) To this hot solution is added slowly, with vigorous stirring with a glass rod, 10 grams of hydroxypropyl methyl cellulose of 15 centipoises nominal viscosity. The methyl cellulose will slowly go into solution as the buffer solution cools. This is methyl cellulose buffered stock solution.

EXample II. To 100 milliliters of water there is added 4.0 grams of polyvinyl pyrrolidone, molecular weight approximately 44,000. This mixture is stirred and shaken until solution is complete.

To 20 milliliters of distilled water there is added 1.0 gram of Wright's Stain Powder. A microprobe ultrasonic transducer is inserted into the mixture and driven at 60 watts output for five minutes. The resulting solution is filtered through Whatman No. 1 filter paper. (The ultrasonic apparatus actually used in this operation by the applicant is known under the Trade Name Sonifier and identified as the Sonifier Cell Disruptor Model No. W-185 of Heat Systems-Ultrasonics, Inc. of Plainview, N.Y.)

Equal volumes of the polyvinyl pyrrolidone solution and of the Wright's stain solution are mixed, and 0.5 milliliters of the mixture are added to 1.0 milliliters of methyl cellulose buffered stock solution.

Example III. To 20 milliliters of N, N,-dimethyl formamide there is added slowly, with stirring, 1.0 grams of Wright's Stain Powder. This is allowed to stand overnight (i.e. about 16 hours) with frequent agitation (which may be provided by a mechanical agitator equipped with a cyclic timer) and then is filtered through Whatman No. 1 filter paper. Of the filtrate 1.0 milliliters are added to 1.0 milliliters of methyl cellulose buffered stock solution.

Example IV. Wright's stain is available in solution in methanol, with a stain content of approximately 0.17 percent by weight; 0.5 milliliters of this solution and 0.5 milliliters of reagent glycerine are added to 1.0 milliliters of methyl cellulose buffered stock solution.

Example V. To 20 milliliters of N, N,-dimethyl formamide there is added slowly, with stirring, 1.0 grams of Giemsa Stain Powder, commission certified, a commercially available product ordinarily used as a convenient form of the solids which, in solution, constitute Giemsa stain. The mixture is allowed to stand overnight (i.e. about 16 hours) with frequent agitation. The solution is filtered through Whatman No. 1 filter paper. Of this filtrate, 1.0 milliliters are added to 1.0 milliliters of methyl cellulose buffered stock solution.

Referring now to Figure 1, there is represented a microscope slide 10 bearing a blood sample, smeared upon it and air dried in conventional fashion, marked 12. A thin bead 14 of any one of the five formulations described above is deposited upon the smeared sample. This may conveniently be done with a small polyethylene "squeeze bottle" equipped with a nozzle similar to that on a conventional oil can. The bead 14 should be slightly shorter than the cover slip.Figure 2 represents a cover slip 16 about to be placed upon the bead 14. When this is done, the surface tension of the bead formulation draws the cover slip down upon the sample smear 12, against the microscope slide 10. The dye dissolved in the formulation of bead 14 is conveyed to the sample smear 12, staining it. The final result appears in Figure 3. The mass marked 18 represents the dye-stained sample smear 12. The formulation which was the bead 14 has now spread to the edges of the cover slip 16, encapsulating the stained sample 18, and has sealed it against the entrance of ambient air, including any moisture in the air.

The five examples given are preferred embodiments. However, it is evident that there are many transparent resins which can be substituted for the particular preferred resin given in the examples, and that a number of different solvents may be substituted in different combinations for those mentioned in the preferred examples. There are certain general considerations which must be taken into account in making other formulations.

It is necessary to use formulations which do not distort or destroy the biological cells being stained, either immediately, or during the comparatively long after period when

the fixed preparation is stored and the last traces of volatile solvent slowly pass off. If the concentration of water in the formulation is high, the rate of diffusion into the cell will usually be high. this ordinarily tends to produce deeper staining, which tends to produce greater stability of the staining in the preparation; but it also tends to produce an osmotic pressure in the cell which will distort, and may actually burst, it. Therefore it is desirable that the liquids in the formulation be a mixture of organic or other nonagueous solvents and water. the examples given range from 25 percent water in No. 1 to 100 percent water in No. 2, with the remainder at 50 percent water.

Example 2 is apparently exceptional to the general rule; for it has been found experimentally that it is not destructive of the cells by excessive diffusion of water into them. The reason for this exceptional behaviour is not known. The molecular weights of the resins involved would appear to be so high that they would not produce an appreciable reduction in the water vapor pressure in the solution; but it is conceivable that the viscosity of the mixture of methyl cellulose and polyvinyl pyrrolidone is sufficient to inhibit the flow of water into the cell walls. This is, of course, pure hypothesis.

Resins such as the alkyl celluloses may be prepared with a wider range of nominal viscosities. These nominal viscosities are ordinarily the voscosity produced in a standard solvent by a standard weight concentration of the sample. If the solubility of a given sample is high, at the standard test concentration it will produce a relatively low viscosity. For use in the present invention, a formulation viscosity is required which will produce suitable performance in the procedure described. A formulation of the required viscosity, if made with a low nominal viscosity resin will contain a higher weight concentration of that than would a formulation of the same viscosity made with a high nominal viscosity resin. When such a formulation dries, by evaporation of the volatile solvents, there will remain the solids actually present plus any nonvolatile solvents (known in the plastics art as "plasticizers"). If a large weight of solids remains, it will occupy a larger volume than a small volume would; this necessarily implies that it will have shrunk less in drying. So it is generally desirable to choose relatively low nominal viscosity resins, in order that they may shrink less and so distort the cells of the sample less. For similar reasons, the incorporation of plasticizers (such as the glycerine in some of the formulations) which will leave the solid resin somewhat flexible is desirable.

There are some general principles which may be used in making formulations, or correcting them. It must be remembered that the art of staining and the art of formulating plastic lacquers or cements are both somewhat empirical; even prepared lacquers and paints bear label instructions for thinning them to suit particular conditions of application. The known properties of stains applied by conventional prior art methods may be taken as a first approximation to their properties when used in the viscous plastic resin solutions taught herein. Buffering to control pH may be accomplished by the use of conventional buffers (cf/McIlvaine's buffer) even though they may, as a matter of convenience, be incorporated in the resin stock solution to which the stain is later added, as in Example 1.

Essentially, the formulation of a viscous fluid stain mixture for the practice of this invention may be carried out in the following steps:

1. The desired stain is selected. This is definitely a function of the user of the invention.

2. Requirements such as concentration/pH control, and any resultant needs for additives such as buffers are established. These, too, depend upon the user and the prior art knowledge of the stains.

3. From data on the solubility of the stain in various solvents a suitable stain solvent is chosen, and the required concentration of stain in it to produce the desired final concentration in the final formulation is determined.

4. The resin is selected. It should be highly transparent, with a refractive index reasonably close to that of glass to minimize reflections at its interface with the cover slip. It should be as soluble as possible, preferably in solvents compatible with the presence of a moderate concentration of water (in the examples given, the concentration of water ranges from about 25 to 67 percent).

5. From the known data on the solubility of the stain and the resin in various solvents, it is determined which solvent to use for the stain and which for the resin. Separate solutions of each may be prepared, and then mixed in the calculated proportions to yield the desired stain concentration and, for a first approximation, a water content of about 50 percent. Viscosity may be adjusted by adding solvent to reduce it. A plasticizer recommended by the resin manufacturer may be added, preferably toward the upper bound of the recommended concentration, since it is desired not merely to avoid cracking or crazing of the solidified resin, but to avoid stresses in it.

6. A test of the initial formulation may be made. If staining is too faint, the water proportion may be increased, or the stain

concentration may be increased. If hoped for differentiation is not obtained, pH adjustment is a logical expedient to try. It must be recognised that the possible problems here considered have to do essentially with the characteristics of the stain used; a stain used in the present invention will not ordinarily produce any better effects than it does when used conventionally.

Surface tension is ordinarily not a problem, since clean glass tends to be wet by many solvents for the transparent resins. If surface tension is only marginally adequate, a reduction in viscosity will usually permit the cover slip to be drawn down as required.

The resins tested with positive results including polyoxyethylene products used commercially primarily as nonionic surfactants, such as the Triton brand of Rohm and Haas, Tween brand of Hercules Powder Company, IGEPAL-CA brand of General Aniline and Film Corporation, and Neuitronyx 600 brand of Onyx Chemical.

Primary solvents tested with positive results include water, dimethyl formamide, glutaraldehyde, methyl alcohol, ethyl alcohol, benzene, toluene, ethyl acetate, and ethylene dichloride.

Stains used include methylene blue NN, cresyl violete acetate, Giemsa stain, Wright stain, May-Grunwald stain, MacNeal tetrachrome stain, methyl green, Pyronin Y, and Jenner's stain.

Plasticizers used include glycerine, polyvinyl pyrrolidone, ethylenediaminetetraacctic acid, and glutaraldehyde.

WHAT WE CLAIM IS:-

1. A method of staining and sealing a biological sample air-dried and affixed to a microscope slide which comprises the steps of:

a) providing a viscous fluid formulation of a transparent resin selected from the group consisting of: methyl cellulose; polyvinyl pyrrolidone and methyl cellulose; ethyl cellulose; carboxymethyl cellulose; hydroxyethyl cellulose; hydroxypropyl cellulose; polyoxyethylene resins: and a biological stain in a solvent mixture comprising water;

b) applying a quantity of the viscous fluid formulation to an area of the affixed biological sample smaller than the area of a cover slip;

c) applying the cover slip to the applied quantity of viscous fluid formulation on the slide;

d) leaving the cover slip undisturbed upon the said fluid formulation on the slide to permit the surface tension of the fluid to draw the cover slip against the sample and the slide, whereby the fluid formulation is spread over the portions of the sample and the slide which are covered by the cover slip, and the stain dissolved in the fluid formulation is brought into contact with the sample and stains it.

2. A viscous fluid formulation for the simultaneous staining and sealing to a coverslip of a biological sample affixed to a microscope slide comprising:

a) a transparent resin selected from the group consisting of methyl cellulose; polyvinyl pyrrolidone and methyl cellulose; ethyl cellulose; carboxymethyl cellulose; hydroxyethyl cellulose; hydroxypropyl cellulose; polyoxyethylene resins

b) a biological stain

c) a solvent comprising water.

3. A viscous fluid formulation as claimed in claim 2 in which the solvent comprises from twenty-five to fifty percent water, by volume .

4. A viscous fluid formulation as claimed in claim 2 or claim 3, in which the solvent comprises a plasticizer for the transparent resin.

5. A viscous fluid formulation as claimed in claim 2, in which

a) the transparent resin is hydroxypropyl methyl cellulose;

b) the biological stain is Wright's stain;

c) the solvent comprises N, N,-dimethyl formamide, water, and glycerine;

the proportions being:

Two parts by volume of N, N-dimethyl formamide having dissolved in it 50 milligrams, per milliliter of N, N,-dimethyl formamide, of hydroxypropyl methyl cellulose of 15 centipoises nominal viscosity;

One part by volume of water having dissolved in it 50 milligrams, per milliliter of water, of Wright's Stain powder; and

One part by volume of reagent grade glycerine.

6. A viscous fluid formulation as claimed in claim 2, in which

a) the transparent resin is a mixture of hydroxypropyl methyl cellulose and polyvinyl pyrrolidone;

b) the biological stain is Wright's stain;

c) the solvent consists essentially of water;

the proportions being:

Four parts by volume of McIlvaine's standard buffer solution having dissolved in it 100 milligrams, per milliliter of standard buffer solution, of hydroxypropyl methyl cellulose of 15 centipoises nominal viscosity;

One part by volume of an aqueous solution of 40 milligrams per milliliter of polyvinyl pyrrolidone of molecular weight approximately 44.000; and

One part of an aqueous solution of 50 milligrams per milliliter of Wright's Stain powder.

7. A viscous fluid formulation as claimed in claim 2, in which

a) the transparent resin is hydroxypropyl methyl cellulose;

b) the biological stain is Wright's stain;

c) the solvent comprises N, N,-dimethyl

formamide and water;
the proportions being:
One part by volume of McIlvaine's standard buffer solution having dissolved in it 100 milligrams, per milliliter of standard buffer solution, of hydroxypropyl methyl cellulose of 15 centipoises nominal viscosity; and
One part by volume of N, N,-dimethyl formamide having dissolved in it 50 milligrams, per milliliter of N, N,-dimethyl formamide of Wright's Stain powder.

8. A viscous fluid formulation as claimed in claim 2, in which
a) the transparent resin is hydroxypropyl methyl cellulose;
b) the biological stain is Wright's stain;
c) the solvent comprises methanol, glycerine, and water;
the proportions being:
Two parts by volume of McIlvaine's standard buffer solution having dissolved in it 100 milligrams, per milliliter of standard buffer solution, of hydroxypropyl methyl cellulose of 15 centipoises nominal viscosity;
One part by volume of a solution in methanol of 0.17 percent by weight of Wright's stain; and
One part by volume of reagent grade glycerine.

9. A viscous fluid formulation as claimed in claim 2, in which
a) the transparent resin is hydroxypropyl methyl cellulose;
b) the biological stain is Giemsa stain;
c) the solvent comprises N, N,-dimethyl formamide and water;
the proportions being:
One part by volume of McIlvaine's standard buffer solution having dissolved in it 100 milligrams, per milliliter of standard buffer solution, of hydroxypropyl methyl cellulose of 15 centiposes nominal viscosity; and
One part by volume of N, N,-dimethyl formamide having dissolved in it 50 milligrams, per milliliter of N, N,-dimethyl formamide, of Giemsa Stain powder.

10. A method of staining and sealing a biological sample air-dried and affixed to a microscope slide as claimed in claim 1, substantially as hereinbefore described with reference to and as illustrated in the accompanying drawings.

11. A method of staining and sealing a biological sample air-dried and affixed to a microscpe slide as claimed in claim 1 substantially as hereinbefore described in any one of the Examples.

12. A viscous fluid formulation for the simultaneous staining and sealing to a cover slip of a biological sample, substantially as hereinbefore described with reference to and as illustrated in the accompanying drawings.

13. A viscous fluid formulation for the simultaneous staining and sealing to a cover slip of a biological sample, substantially as hereinbefore described in any one of the Examples.

14. A slide when produced by a method as claimed in any one of claims 1, 10 and 11.

J.A. Bleach,
Agent for the Applicants

Printed for Her Majesty's Stationery Office,
by Croydon Printing Company Limited, Croydon, Surrey, 1979.
Published by The Patent Office, 25 Southampton Buildings,
London, WC2A 1AY, from which copies may be obtained.

1557722 COMPLETE SPECIFICATION
1 SHEET *This drawing is a reproduction of the Original on a reduced scale*

Fig. 1.

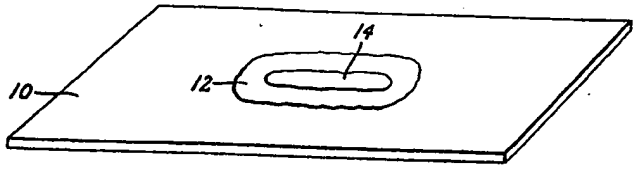

Fig. 2.

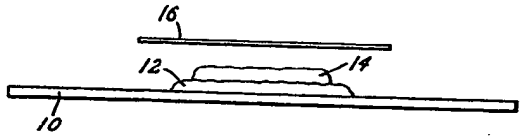

Fig. 3.